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Hydantoin-hydrolysing enzymes for the enantioselective production of amino acids: new insights and applications

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Abstract—This report describes recent discoveries and new applications in the field of hydantoinase biocatalysis with particular attention to stereoselective reactions and new mechanistic understanding of stereocontrol as well as new enantiopure products and novel applications. Hydantoin-hydrolysing systems comprise hydantoinase and *N*-carbamoylamino acid amidohydrolase (NCAAH) enzymes, which sequentially catalyse hydrolytic hydantoin ring opening and hydrolytic cleavage of *N*-carbamoyl groups, respectively, yielding enantiopure amino acids, which may be non-natural, and may have the L- or D-configuration.

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1. Introduction

The biocatalytic conversion of hydantoins to amino acids has been recognised in recent decades for potential application in the industrial production of amino acids, both natural and non-natural. These serve as important synthons for the production of pharmaceuticals (e.g., semi-synthetic penicillins and cephalosporins), agrochemicals and fine chemicals. The biocatalysis reaction involves two consecutive hydrolysis steps, catalysed by an hydantoinase (dihydropyrimidinase EC 3.5.2.2) and *N*-carbamoylamino acid amidohydrolase (*N*-carbamoylase; EC 3.5.1), respectively. In the conversion, an L- or D-selective or non-selective hydantoinase converts a 5-monosubstituted hydantoin to an L- or D- (or mixture of L- and D-) *N*-carbamoylamino acid. An L- or D-selective NCAAH then converts the *N*-carbamoylamino acid to an L- or D- (or mixture of L- and D-) amino acids. Both hydantoinase and NCAAH activity may be present in one microorganism, but some possess only one or other of the activities.[1] An analysis of stereoselectivity in relation to the microbial source has been reported.[2] In some microorganisms, the conversion of hydantoins to *N*-carbamoylamino acids and amino acids is accompanied by the interconversion of D- and L-hydantoins, mediated by racemases. The presence of racemases in hydantoin cleaving systems is variable, but may be advantageous in some cases.[3,4] Rapid racemisation of many hydantoins can allow up to 100% conversion of

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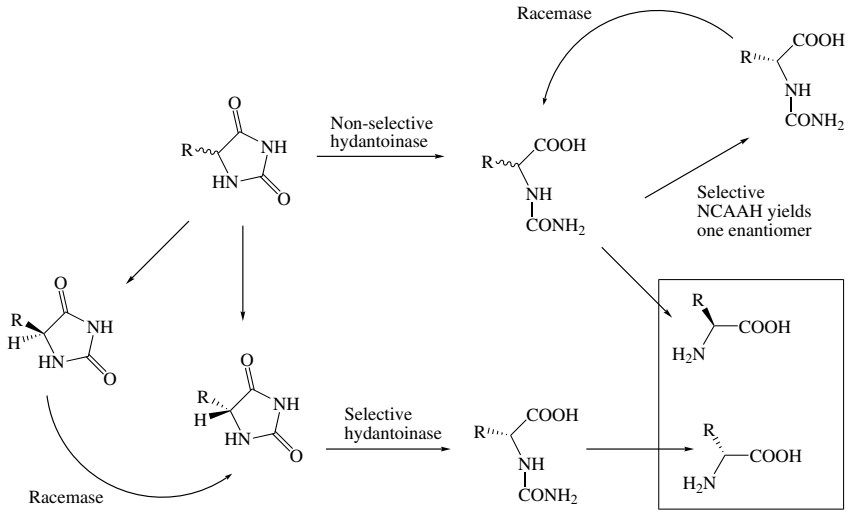

Figure 1. Variation in stereoselectivity in hydantoin-hydrolysing systems in different microbial systems. Selective or non-selective hydantoinase or NCAAH activity leads to differing product stereochemistry.

racemic hydantoins to enantiomerically pure products in one step, and the yield can also be increased through chemical racemisation of unconverted enantiomers.

Thus, the stereoselectivity of the reaction system may arise from the activity of either the hydantoinase or the NCAAH, or both or neither (Fig. 1). Furthermore, it may also be substrate dependent, and the role of the racemase has until recently been unclear. A number of microorganisms that produce both hydantoinase and NCAAH have been described in literature, many of which are D-selective. While L-selective systems are less common, enantiomerically pure L-amino acids are of great commercial value particularly with respect to pharmaceutical applications.[5] As an example, *Pseudomonas putida* strain RU-KM3s, has high levels of hydantoin-hydrolysing activity, with non-stereoselective hydantoinase and L-selective *N*-carbamoylase activity.[6,7]

This variability among hydantoin-hydrolysing systems has lead to an extensive literature describing enzymes from a wide range of sources. The mechanisms for stereo chemical control are only currently being elucidated, aided by recent access to crystal structures, recombinant proteins including those produced by rational design, and computational techniques including in silico substrate docking experiments. The current drive in hydantoinase technology is the development of biocatalysts where both activities are present with high levels of the desired stereoselectivity as well as stability suited to process conditions. This report seeks to highlight recent developments towards these objectives.

2. Molecular and biochemical characterisation of hydantoin-hydrolysing systems

The regulation of hydantoinase activity is complex and has been the subject of recent study. The ability to utilise hydantoins as sole nitrogen sources is a well-recognised means of selecting for hydantoin-hydrolysing strains. In some cases the hydantoins are utilised only under conditions of nutrient stress or catabolite repression, as exemplified by Hartley et al.[8] for an *Agrobacterium tumefaciens* strain. A study identifying the hydantoinase and *N*-carbamoylase-encoding genes in *P. putida* RU-KM3s, using transposon mutagenesis and selection for altered growth phenotypes, showed that inactivation of the genes encoding a dihydropyrimidinase and β-ureidopropionase resulted in loss of hydantoinase and *N*-carbamoylase activity. Transposon mutants in which the nitrogen regulatory pathway was disrupted retained the dihydropyrimidinase or β-ureidopropionase activities, but disruption of ketoglutarate metabolism resulted in a significant reduction in the activity of both enzymes, suggesting that carbon catabolite repression was involved in the regulation of hydantoin hydrolysis in this strain.[9]

Crystal structures of hydantoinases have only recently been reported; for example, that of the hydantoinase of *Bacillus stearothermophilus* SD1 was reported by Cheon et al.[10] to have structural similarity and a similar hydrolytic mechanism to that of the enzyme dihydroorotase but the two enzymes differed in modes of substrate recognition. This can inform further work on directed evolution to evolve hydantoinases with novel substrate specificities. A more in-depth analysis of the protein structure of this enzyme by the same authors has highlighted the role of three loops (stereochemistry gate loops), which form a hydrophobic substrate binding pocket in determining the stereoselectivity of D-hydantoinases.[11]

Yoon et al.[12] recently reported modification of an hydantoinase to a dimeric form with altered activity. The paper included a survey of the molecular characteristics of four groups of amide-hydrolysing enzymes including hydantoinases, and discussed the structural relationships between hydantoinases and dihydropyrimidinases, which involve the formation of dimers and tetramers of the proteins by hydrophobic interactions, relating enzy-

matic activity to higher oligomeric states. Where oligomeric association was found, the activity was shown to be controlled via allosteric regulation, in contrast with monomeric proteins where rates were controlled by non-allosteric mechanisms.

In one of the very few papers reporting characterisation of a hydantoin racemase, Las Heras-Vázquez et al.[13] recently reported the cloning and expression of the hydantoin racemase gene from *A. tumefaciens* C58 in *Escherichia coli* BL21, producing an active enzyme with L-specificity. While the native protein was a tetrameric protein, this racemase was isolated as a monomer, but its activity and kinetic properties were similar to others reported.

New variants of hydantoinase enzyme systems continue to be discovered and characterised; for example, the hydantoinase from *A. tumefaciens* BQL9 was recently reported to be very similar to numerous other hydantoinases from this species.[14] More interesting, however, are new enzymes discovered in novel species, as for example the thermostable oligomeric hydantoinase reported to have been isolated from a hyperthermophile, *Methanococcus jannaschii*, which grows optimally at 85 °C. The hydantoinase was cloned and expressed in *E. coli*, and shown to be L-specific using 5-hydroxymethylhydantoin as substrate.[15]

Xu et al.[16] reported the crystal structure of D-hydantoinase from the mesophile *Burkholderia pickettii*, which showed limited sequence homology with hydantoinases from a *Thermus* sp. and *B. stearothermophilus* but significant similarities in the structure of the catalytic active site. On the basis of protein structure studies, a molecular basis for thermostability of D-hydantoinases was proposed where hydrophobic and bulky residues are more predominant in the interiors of the thermostable enzymes, and more capacity for salt bridges and hydrogen bonding, and less potential for oxidation, would preserve thermostability.

3. Mechanistic and kinetic studies of hydantoin-hydrolying systems

The catalytic mechanism of hydantoinases has received recent attention with the particular objective of understanding the stereoselectivity. A catalytic mechanism was proposed to explain the D-selectivity of the hydantoinase from *Vigna angularis*, based on its ability to convert (*R*)-5-substituted hydantoins but not 5,5-disubstituted derivatives.[17] This kinetic study showed that the substrate must have a hydrogen in pro-*S* face in order for hydrolysis to occur, and suggests that rapid chemical racemisation of the substrate must take place before hydrolysis can be achieved (Fig. 2).The crystal structure of the dihydropyrimidnase (D-hydantoinase) from a *Thermus* sp. was solved showing that the hydantoinase has some homology with ureases, dihydroorotases and phosphotriesterases. In silico docking experiments were conducted and gave support for the proton-dependent mechanism.[18]

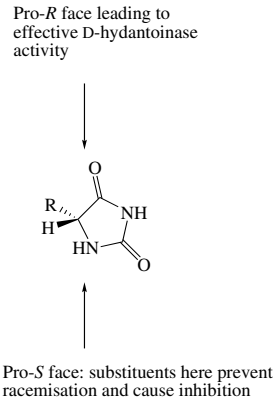

Figure 2. Proposed explanation for D-specific activity in hydantoinase from *Vigna angularis* (adapted from Arcuri et al.[17]).

The kinetic analysis of biocatalytic reaction systems involving two biochemically different enzymes, often with widely differing rate characteristics, is complex and can usefully be aided by mathematical kinetic modelling as a means of optimising the reaction productivity. This in turn facilitates process design to accommodate the differing rates and reaction conditions required by hydantoinase and NCAAH, respectively. Some attention has been given to development of biocatalysts using the two activities derived from different microorganisms. In our laboratory we have recently measured the kinetics of hydantoin conversion using immobilised enzymes from *P. putida* RU-KM3s and *A. tumefaciens* RU-OR, modelled the production of amino acids, and used the kinetic model successfully to optimise the productivity of a combined biocatalyst comprising both enzymes in a customised continuous reactor (Bulawayo et al., personal communication). Similarly, a simulated kinetic model was developed for the production of D-*p*-hydroxyphenylglycine from D,L-*p*-hydroxyphenylhydantoin using the D-hydantoinase of *B. stearothermophilus* SD1 and NCAAH of *A. tumefaciens* NRRL B11291.[1] In the latter study, the enzymes were used in whole cell systems, either singly or co-expressed in the same cells, demonstrating the feasibility of a recombinant approach.

4. Applications of hydantoin-hydrolysing systems

In bioprocesses, the enzymatic conversion of substituted hydantoins to amino acids can be accomplished in one step if both enzymes needed for the biotransformation are present. Kim and Kim[19] achieved a one-step enzymatic production of D-*p*-hydroxyphenylglycine (D-HPG) from 5-substituted hydroxyphenylhydantoin (HPH) using the strain *Agrobacterium* sp. I-671, which possessed both D-hydantoinase and an *N*-carbamoylase activity. However, in such multi-enzyme systems, the activity of one of the enzymes can be limiting, and modification to improve activity or selectivity may only be partly successful. For instance, in a study to improve D-HPG production from D-HPH using a recombinant *E. coli*, the D-hydantoinase activity was increased

2.57-fold but carbamoylase activity remained constant, resulting in only 30% increased reaction rate.[20]

The search for new reaction systems capable of producing novel amino acid derivatives has recently yielded a route to (*S*)-(+)-2-amino-4-phenylbutanoic acid from racemic 5-[2-phenylethyl]-imidazolidine-2,4-dione (*rac*-**2**) using a non-stereoselective hydantoinase and an L-specific NCAAH expressed in a *Bacillus* spp. This is one of the first reports of application of a fully recombinant hydantoin-hydrolysing reaction system.[21]

Synthetic routes to non-natural amino acids were reviewed by Altenbuchner et al.,[22] where the significance of progress in the development of heterologous enzyme expression and enzyme evolution for improved production systems are well described. The range of substrates, which can be converted has been extended to include, for example, non-polar silicon compounds (dimethyl)phenylsilyl- and 1-methyl-1-silacyclopentyl substituted alanine derivatives, which were converted by hydantoinase and L-specific NCAAH enzymes.[23] Arcuri et al.[24] also successfully synthesised *N*-carbamoyl-D-*p*-fluorophenylglycine and *N*-carbamoyl-D-*p*-trifluoromethylphenylglycine using the D-hydantoinase from *V. angularis*. The D-selective hydantoinase from *Blastobacter* sp. A17p-4 was used for hydrolysis of cyclic imides and amides, illustrating the broad substrate selectivity of some hydantoin-hydrolysing systems and suggesting that their metabolic function resembles that of dihydropyrimidinases in pyrimidine metabolism.[25]

The isolation and purification of hydantoin-hydrolysing enzymes is not necessarily straightforward. An alternative approach in biocatalysis applications has been to use a 'multi-enzyme extract', which contains predominantly the hydantoinase and NCAAH activities derived from the selected bacterial strain. This approach can yield effective biocatalysts in terms of hydantoin conversion to amino acid product but the stereoselectivity of the system may be limited by the enantioselectivity of the least enantiospecific of the two enzymes, and in strains where one of the enzymes is stereoselective and the other in non-specific, the stereoselective component activity needs to be rate-controlling to ensure enantiopure products.

An extract containing both the D-hydantoinase and the D-NCAAH from *Agrobacterium radiobacter* was immobilised on chitin and used for production of *p*-hydroxyphenylglycine.[26] Similarly, Ragnitz et al.[27] conducted a comprehensive investigation of hydantoin-hydrolysing biocatalysts from *Arthrobacter aurescens* DSM 3747 and 3745, using several immobilisation methods, which included both crude extracts and purified and recombinant enzymes. Immobilisation on Eupergit and Sepharose supports, where covalent coupling via oxirane or carbodiimide or linkage involves amino, thiol and hydroxy groups or carboxylic acid groups, respectively, were compared. While the (non-enantioselective) hydantoinase activity was readily immobilised on Eupergit C 250 L as the best support, to give a stable biocatalyst, the crude L-specific NCAAH activity was found to be lost under conditions other than carbodiimide linking of carboxylic acid groups. Purified recombinant L-NCAAH used to investigate this effect. The purified L-NCAAH was shown to be more successfully immobilised on the more hydrophilic support EAH Sepharose, and very high activities were reported.

Immobilisation can lead to enhanced enzyme activities for varied reasons, and this has been reported for hydantoin-hydrolysing systems. The NCAAH activity in a crude extract of *A. tumefaciens* RU-OR was shown to have greatly enhanced activity and stability when immobilised in calcium alginate.[28] Similarly, the hydantoinase activity showed a 5-fold increase in activity relative to the non-immobilised hydantoinase. In the immobilised biocatalyst, the hydantoinase and *N*-carbamoylase activities remained more stable in storage than the non-immobilised enzymes. While this support is not necessarily suited to large scale applications, we have observed similar effects using alternative supports (Burton et al., unpublished results) and others have reported similar results.[26]

5. Conclusion

While hydantoinase technology is now well established, the field continues to generate new synthetic products of interest and to provide new insights into biocatalytic enzyme function.

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